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micrograms

10 to the 6th power

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Influence of Cadmium, Nickel, and Chromium on Primary Immunity in Mice[1]

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The effects of metals on the primary humoral immune system of mice were investigated using a hemolytic plaque technique to determine the number of specific antibody-producing spleen cells. Inhalation of $NiCl_2$ for 2 hr resulted in a significant negative linear dose response, the lowest effective concentration tested being 250 μg of Ni/m^3. Following a 2 hr aerosol exposure to $NiCl_2$, the lung cleared Ni on a first-order kinetics basis. A significant reduction in the number of plaques per 10^6 cells also was observed with exposure to 190 μg of Cd/m^3. Analyses of the data from intramuscularly exposed mice indicated that concentrations greater than or equal to 3.90 μg of Ni/g body weight (as $NiSO_4$) and greater than or equal to 9.25 μg of Ni/g body weight (as $NiCl_2$) resulted in significant immunosuppression. Intramuscular treatments with NiO, $CdCl_2$, and $CrCl_3$ had no effect at the concentrations tested.

INTRODUCTION

Evidence now exists which indicates that exposure to a number of trace metals can significantly alter the functioning of host defense mechanisms. Waters *et al.* (1975) and Graham *et al.* (1975a) have demonstrated that alveolar macrophages, which significantly aid in defense of the deep lung, are reduced in number, lose viability, and show decreases in phagocytic and enzymatic activity following exposure to Cd, Ni, Cr, Mn, and V. In the upper airway, metals may exert their toxic effect by altering the functioning of the mucociliary escalator. Both *in vivo* and *in vitro* exposure to Cd (Adalis *et al.*, 1977) and Ni (Adalis, personal communication, 1977) cause a reduction in ciliary activity in hamster tracheal rings. The net effect of these various insults on host defense systems is reflected as an enhancement of mortality in mice exposed to aerosols of Cd, Ni, and Mn (Gardner *et al.*, 1976; Graham and Gardner, 1977; Maigetter *et al.*, 1976; Adkins and Gardner, 1976) prior to a challenge with infectious microorganisms.

The three metals, Cd, Cr, and Ni, which were chosen for this study, are industrial and environmental pollutants that are associated with respirable-sized particles (Natush *et al.*, 1973; Lee and Von Lehmden, 1973). In view of the adverse health effects of these metals, systemic protective systems were investigated by examination of primary humoral immunity following pollutant exposure.

[1] Disclaimer statement: This report has been reviewed by the Office of Research and Development, EPA, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Environmental Protection Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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MATERIALS AND METHODS

Animals. Six-week-old, specific pathogen-free, female, Swiss albino mice, strain CD-1 (Charles River Farms, Inc., Wilmington, Massachusetts), weighing 20 to 25 g were used for all experiments.

Intramuscular metal exposures. In these studies, the following metallic salts were used without further purification: $CdCl_2$ (Matheson, Coleman and Bell, Norwood, Ohio), $CrCl_3$ (J. T. Baker Chemical Co., Phillipsburg, New Jersey), $NiCl_2$ (International Nickel Co., Inc., Suffern, New York), $NiSO_4$ (Fisher Scientific Co., Raleigh, North Carolina), and NiO (a water-washed preparation, courtesy of Dr. Michael Waters, EPA).

For the intramuscular (IM) exposures, the test substances were prepared by mixing appropriate amounts of the metals in Dulbecco's saline (all media were obtained from GIBCO, Grand Island, New York). The animals were exposed on a micrograms of metal per gram body weight basis. The concentrations of metals in the diluent were adjusted so that each mouse received an injection of approximately 0.25 ml.

To determine the method for preparing a metal solution used for IM exposure, mice were injected with either a filtrate or an unfiltered solution of NiO or $NiSO_4$. To obtain the filtrate, the metals were allowed to remain in the saline for 25 hr prior to passing an aliquot of each sample through a 0.2-μm filter (Millipore Corp., New Bedford, Massachusetts). Concentrations of 3.09 and 12.34 μg of Ni/g body wt were tested.

Since the higher concentrations of some metals, particularly Cr, are known to be more acidic, the influence of pH in this model system was also tested. Dulbecco's saline was adjusted to pH 4 or 6 with either HCl or acetic acid. The mice then received IM injections of 0.25 ml of these fluids. A group of control mice injected with Dulbecco's saline (pH 7) was also tested.

Aerosol metal exposures. Mice were exposed to aerosols of $NiCl_2$ or $CdCl_2$ for 2 hr. A complete description of the exposure methods has been given by Gardner *et al.* (1976). Animals are placed into individual containment modules which are then inserted into a rectangular Plexiglas exposure chamber that allows only the heads of the animals to be exposed. Particles are generated using a modified fluid atomization aerosol generator (Environmental Research Corp., St. Paul, Minnesota). Using an Aerosol Particle Monitor (Royco Instruments, Palo Alto, California), it was determined that 99% of the particles were less than or equal to 3 μm in diameter. For $CdCl_2$ at least 99% of the particles were less than or equal to 3 μm in diameter. Determinations of actual metal concentrations were made by atomic absorption spectrophotometry.

Immunologic procedures. The methods of immunization and the hemolytic plaque assay used have been described in detail elsewhere (Graham *et al.*, 1975b). Briefly, all animals, including controls, are immunized with 0.5 ml of a sheep red blood cell (SRBC) suspension ($4.5-5 \times 10^8$ SRBC/ml) injected intraperitoneally immediately after the IM metal injection (except where indicated) or 2 hr following aerosol exposure. This time is referred to as "Day 0." A direct Jerne plaque technique is used to test the IgM antibody-producing capability of spleen cells harvested on the fourth day after immunization. For the experiments described

here, cells from each individual mouse were plated in triplicate. The number of plaques per plate was converted to the number of plaques per 10^6 cells for analysis. Since preliminary experiments showed that the viability of the cells as determined by trypan blue dye exclusion, was not affected by the metal treatments, no adjustment of the cell counts was necessary.

Clearance of Ni from the lung and spleen. For the clearance studies, mice were exposed to a $NiCl_2$ aerosol of 644 μg of Ni/m^3 for 2 hr. Immediately following exposure and at 24-hr intervals thereafter, mice were sacrificed and their lungs and spleens were removed and weighed. The organs were ashed, dry weights were determined, and the concentration of Ni was assayed using atomic absorption spectrophotometry.

Experimental design and statistical methodology. A combination of regression and analysis of variance (ANOVA) techniques was used in the analyses of these experiments. Significant daily variation in the mean number of control plaques per 10^6 cells was accounted for via a blocking factor, which was in accordance with earlier work (Graham *et al.*, 1975b). A logarithmic transformation was applied to the data, since the standard deviation of the number of plaques per 10^6 cells was found to be proportional to the mean response level.

The time of maximal difference in plaque production between pollutant-exposed and control animals with respect to the day of immunization was examined using Dunnett's test (1955). For the testing of possible toxic effects of Ni, Cd, and Cr upon primary antibody production in the spleen, the results from several dose levels for each metal were examined using William's test (1972) to determine the lowest dose at which there was evidence of significant immunosuppression.

When various IM treatment groups were to be compared within an experiment, each replicate included a control group of mice which was injected with 0.25 ml of Dulbecco's saline. The daily replicate was conducted using randomly selected groups of animals, each of which received a particular exposure (various concentrations of one metal, acidic solutions of different pH, or filtered or unfiltered fractions of different metal concentrations).

For the aerosol exposures, logistical restrictions prevented the testing of all metal concentrations on the same day. Therefore, on a given day, control and treatment groups representing one metal concentration were tested. Control animals were placed into the individual containment modules and allowed to breathe room air. Comparisons between metal concentrations were made after expressing the results as the difference from the control.

RESULTS

Development of the Model

Optimization of response differences. Initial studies were conducted to determine if the time of metal exposure relative to the time of immunization affects the toxicity observed. Results obtained using $NiCl_2$ IM injections of 9.25 μg of Ni/g body weight are given in Fig. 1. The largest difference in plaque production between nickel-treated and control mice was found to occur when immunization was on the same day as the metal exposure. This difference was the only one judged to be statistically significant ($P < 0.10$) when multiple comparison tests

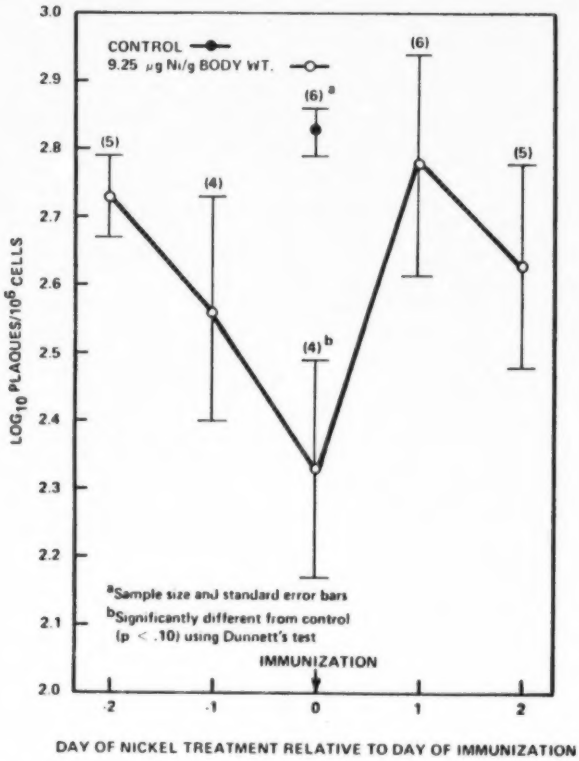

FIG. 1. Plaque production as a function of the day of Ni treatment (relative to the day of immunization).

were performed comparing the various metal exposure days with the control. Based upon these results, all subsequent immunizations were made on the same day as metal exposure.

Effects of pH. Since some of the proposed test substances such as $CrCl_3$ are known to be acidic and since one of the metal concentrations to be used was at pH 5, the effects of pH on plaque production were examined using the experimental design described earlier. No statistically significant differences in plaque production were detected when the treatment groups were considered in general nor when specific subtests concerning the effects of pH and type of acid were made. However, the general level of plaque production did vary significantly over the various days required to complete the experiment. The "Days by Treatment" interaction ($P = 0.1$) was examined by plotting the five treatment means for each of the four replicate days (Fig. 2). No consistent pattern of effects is evident, and the unusually low number of plaques per 10^6 cells for one of the replicates for mice treated with HCl at pH 6 is the apparent cause of the significant interaction. Hence, the data show that injections of fluids having pH's between 4 and 7 are not a critical factor in this model system.

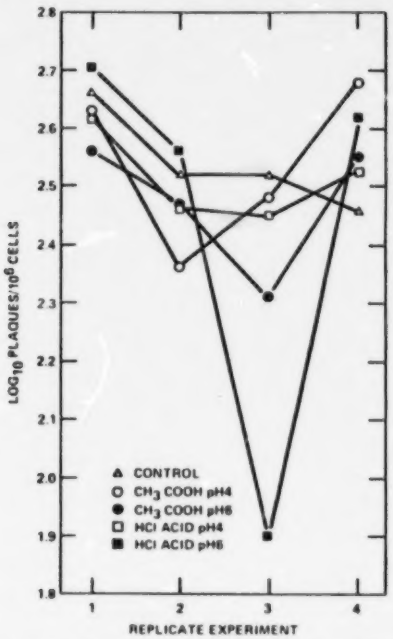

FIG. 2. Treatment means from replicate experiments on the effects of acidity on the number of plaques per 10^6 cells. No consistent trends in the differences between the various treatment groups are evident.

Effects of unfiltered metal solution versus soluble fraction. Comparisons were made between plaque production of cells from mice which had been injected with either a filtrate or an unfiltered solution of NiO or $NiSO_4$. Table 1 gives the results obtained from these studies and indicates opposite effects for these compounds.

For $NiSO_4$ there was no significant difference in toxicity between the soluble and the unfiltered fractions, but the high concentration significantly depressed plaque production as compared to the low concentration (2.36 vs 2.50 $\log_{10}$ plaques/10^6 cells). Conversely, a significantly lower response was observed with the unfiltered NiO suspension as compared to the filtered solution (2.60 vs 2.76 $\log_{10}$ plaques/10^6 cells), but concentration differences were not detected. Major shifts in the overall level of plaque production from day to day were present in these experiments, as evidence by "Day" being a significant source of variation in Table 1. However, the pattern of treatment differences was consistent from day to day as evidenced by the lack of a significant "Days by Treatments" interaction.

Based on these results, it was decided that animals would receive IM injections of unfiltered metals.

Effects of Intramuscular Exposures

Table 2 gives the data from the $CdCl_2$ and $CrCl_3$ exposure experiments. Increasing concentrations of Cd resulted in a progressive decrease in $\log_{10}$ plaques per 10^6

TABLE 1
SUMMARY OF THE EFFECTS OF FILTRATE AND UNFILTERED FRACTIONS OF TWO NICKEL COMPOUNDS ON SPLENIC PLAQUE PRODUCTION

Source of variation	$NiSO_4$			NiO		
	Degrees of freedom (df)	*F* value	*P*	Degrees of freedom (df)	*F* value	*P*
Days	3	4.52	0.0055	1	24.78	0.0001
Treatments	4	1.55	0.19	4	1.54	0.20
Ni concn (low vs high)[a]	1	4.85	0.03	1	0.79	0.38
Fraction (filtrate vs unfiltered)	1	0.001	0.99	1	4.18	0.047
Ni concn × Fraction	1	0.43	0.52	1	1.29	0.26
Days × Treatments	12	0.75	0.70	4	0.75	0.57
(Error variance, df)		(0.098, 99)			(0.076, 47)	

[a] Low and high denote 3.09 and 12.34 μg of Ni/g body wt, respectively.

cells, but these differences were not significant since the variability in the response with Cd exposure was approximately double that observed with the other compounds. Cadmium concentrations greater than 11.81 μg/g body wt were not tested since this level resulted in approximately 20% mortality. Statistically significant alterations in plaque production were not observed with $CrCl_3$.

Statistically significant ($P < 0.05$) Ni treatment differences were observed in plaque production of mice injected with $NiCl_2$ or $NiSO_4$ (Table 3). The various concentrations of NiO tested did not cause significant immunosuppression. It can also be seen from Table 3 that daily patterns of effects were consistent, although the overall response level varied.

The lowest effective ($P < 0.05$ using William's test) concentrations of $NiCl_2$ and $NiSO_4$ tested were 9.25 and 3.09 μg of Ni/g body weight, respectively. A significant negative linear dose response was observed with $NiCl_2$. However, with $NiSO_4$, once the initial effect was observed, increasing the concentration did not significantly enhance the response.

TABLE 2
DOSE–RESPONSE EFFECTS OF CHROMIUM AND CADMIUM CHLORIDES ON PLAQUE PRODUCTION

$CrCl_3$			$CdCl_2$		
Micrograms per gram body wt	No.	Log_{10} plaques/10^6 cells	Micrograms per gram body wt	No.	Log_{10} plaques/10^6 cells
0.0	6	2.67	0.0	12	2.58
1.37	6	2.80	1.48	12	2.57
2.74	6	2.59	2.96	12	2.47
5.47	6	2.64	5.91	9	2.46
10.94	5	2.46	11.81	9	2.40
	(0.0458, 24)[a]			(0.098, 44)[a]	

[a] Error variance and degrees of freedom, respectively.

TABLE 3
DOSE–RESPONSE EFFECTS OF VARIOUS NICKEL COMPOUNDS ON PLAQUE PRODUCTION

Micrograms of Ni/per gram body wt	Log_{10} plaques/10^6 cells[a]		
	$NiCl_2$	$NiSO_4$	NiO
0.0	2.63	2.86	2.71
3.09	2.57	2.67*	2.60
6.17	2.47	2.53*	2.38
9.25	2.37*	2.64*	2.59
12.34	2.29*	2.63*	2.66

ANOVA

Source of variation	Degrees of freedom (df)	$NiCl_2$		$NiSO_4$		NiO	
		F value	*P*	*F* value	*P*	*F* value	*P*
Ni treatment	4	3.09	0.024	3.71	0.011	1.41	0.245
Day	1	9.05	0.004	19.06	0.0002	70.79	0.000
Day × Ni treatment	4	0.59	0.678	1.40	0.248	0.72	0.587
(Error variance, df)		(0.0745, 48)		(0.0497, 47)		(0.1082, 47)	

[a] Ten to 12 mice in each treatment–dose group.
* Significantly different from control ($P < 0.05$) using William's test.

Effects of Aerosol Exposures

Animals receiving 110 μg of Cd/m^3 ($N = 17$) had an average of 95 ± 17 SE plaques/10^6 cells more than their respective controls ($N = 20$). This difference was not significant. However, mice ($N = 16$) that were exposed to 190 μg of Cd/m^3 had 380 ± 16 SE fewer plaques/10^6 cells ($P < 0.05$) than their controls ($N = 20$).

Linear regression analysis on the number of plaques/10^6 cells (treated − control) of $NiCl_2$-exposed mice showed a significant linear negative dose response ($P < 0.05$) (Fig. 3). When each treatment group was tested against its own control, all metal concentrations greater than or equal to 250 μg of Ni/m^3 produced a significant effect.

In another analysis, the $NiCl_2$ and $CdCl_2$ exposure concentrations were converted to microgram-atoms per cubic meter to compare the effect of an exposure to a certain number of atoms of one metal to the same number of atoms of another metal. On this basis, the concentration of 110 μg of Ni/m^3 (1.87 μg-atoms of Ni/m^3) is similar to 190 μg of Cd/m^3 (1.69 μg-atoms of Cd/m^3). This concentration of Cd caused a greater depression in the number of plaques/10^6 cells than did the Ni exposure ($P < 0.0001$).

$NiCl_2$ Clearance

The Ni burdens of the lung and spleens of mice at various days following exposure are shown in Table 4. There was no difference between examining clearance based upon wet or dry tissue, so analyses were based on micrograms of nickel per gram dry wt. Clearance of Ni from the lung followed first-order kinetics (Fig. 4). After 4 days of recovery in clean air, there was still a significantly

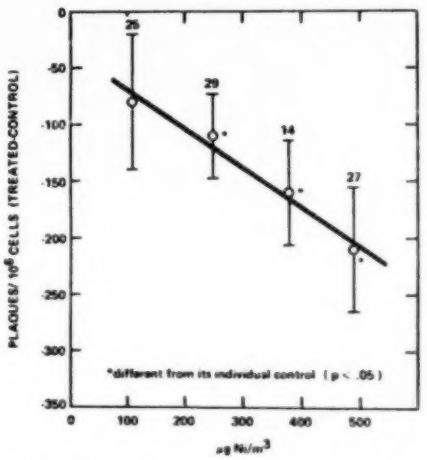

FIG. 3. Dose–response effects of $NiCl_2$ aerosols on the number of plaques per 10^6 cells. The numbers above the standard error bars indicate the number of mice exposed. The predicted equation for the negative linear regression ($P < 0.05$) is given by $\hat{Y} = -34.9 - 0.347X$.

($P < 0.05$) higher concentration of Ni in the lung as compared to the control. Analysis of variance (ANOVA) of the dry wt/wet wt ratios indicated that edemagenesis did not occur in the lung.

ANOVA showed that in the spleen, for all time periods studied, there was no significant increase in the amount of Ni present in exposed animals over control. There was a general elevation in the dry wt/wet wt ratios for the Ni exposed animals over all time periods (Table 4). However, only the increases of 11.4% at

TABLE 4
CLEARANCE FOLLOWING A TWO-HOUR EXPOSURE TO 644 μg OF Ni/m³[a]

Organ	Days postexposure	Micrograms of Ni per gram dry wt	Dry wt/wet wt
Lung	0	8.06 ± 0.506	0.249 ± 0.0051
	1	5.29 ± 0.376	0.197 ± 0.0083
	2	4.05 ± 0.136	0.249 ± 0.0097
	3	3.79 ± 0.173	0.218 ± 0.0061
	4	2.29 ± 0.274	0.266 ± 0.0278
	Control	0.97 ± 0.093	0.213 ± 0.0052
Spleen	0	1.05 ± 0.086	0.261 ± 0.0060
	1	0.86 ± 0.045	0.252 ± 0.0049
	2	0.78 ± 0.065	0.282 ± 0.0098
	3	1.35 ± 0.190	0.252 ± 0.0075
	4	1.29 ± 0.373	0.263 ± 0.0083
	Control	0.89 ± 0.081	0.236 ± 0.0052

[a] The data are given as means ± SE. Four days post exposure means are based upon results using eight animals, while all other means are based upon results using nine animals.

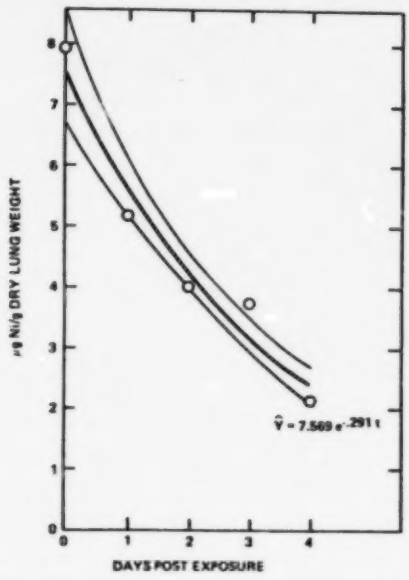

FIG. 4. Clearance of Ni from the lung following a 2-hr exposure to 644 μg of Ni/m³ (as $NiCl_2$). The response curve with its 95% confidence limits indicates first-order clearance kinetics.

Day 2 and 19.5% at Day 4 were judged to be significantly different from the control using Dunnett's test.

DISCUSSION

The factors that were examined in the model development are illustrative of design and analysis problems that must be considered in implementing the model for studying the effects of other pollutants on the humoral immune system. The metals that reduced plaque production may have done so by either direct or indirect means. However, from the experiments conducted here, it is not possible to hypothesize the actual mechanism of action. In any case, regardless of the mechanism, aerosols of $NiCl_2$ and $CdCl_2$ and IM injections of $NiCl_2$ and $NiSO_4$ caused a significant decrease in the number of specific antibody-producing spleen cells.

The results of some of the studies reported here allow speculation about the role of the cation versus the anion in the toxicity of Ni compounds. Approximately three times as much Ni administered in the form of $NiCl_2$ as compared to $NiSO_4$ was required to produce significant immunosuppression. In addition, $NiCl_2$ produced a linear dose–response effect, while the $NiSO_4$ effect was maximal and unchanging beyond 3.09 μg/g body wt. Since equal concentrations of Ni were injected, the differences in results reflect the effect of the anion, with SO_4 exacerbating the toxicity. While both of the Ni salts were effective, the oxide form was not. This may be due to the insolubility of NiO.

A number of investigators have examined the effect of Cd on the immune

system. Exon *et al.* (1975) reported on preliminary work which showed decreased antibody synthesis of splenic lymphocytes of mice exposed to $CdCl_2$ in drinking water. Koller (1973) found that rabbits receiving 300 ppm of $CdCl_2$ in the drinking water for 70 days showed depression in pseudorabies virus-neutralizing antibody titer. In another study, Koller *et al.* (1976) exposed mice to 0.15 mg of Cd either ip or orally by intubation, immunized them with SRBC 8 hr later, and determined the hemolytic plaque response. Only the oral exposure resulted in significant immunosuppression. Thus, the route of exposure plays an important role in the toxicity of this metal for the immune system.

This observation is also supported by the results obtained in the current study. Previously, Graham *et al.* (1975b) showed that IM injections of 9.25 and 12.34 μg of Ni/g body wt produced immunosuppression. The lowest effective dose tested amounted to approximately 208 μg of Ni/mouse. With the same model system, aerosols of $NiCl_2$ greater than 250 μg of Ni/m^3 caused a reduction in plaque response. Based upon a minute volume of 1.45 ml/g body wt (Fairchild, 1972), computations show that exposure to 250 μg of Ni/m^3 would deliver approximately 0.98 μg of Ni/mouse, assuming total retention of all inhaled metal. Thus, the effective dose ratio for these two routes of exposure is approximately 200.

A similar comparison can be made for $CdCl_2$. The highest IM concentration of $CdCl_2$ tested (11.81 μg of Cd/g body wt), which delivered approximately 266 μg of Cd/mouse, caused no significant effects. However, significant immunosuppression resulted from 190 μg of Cd/m^3, which corresponds to an approximate maximum dose of 0.74 μg Cd/mouse. This is an approximate 350-fold dose difference between a noneffective IM and an effective aerosol treatment. Differences in the effective dose may reflect differences in detoxification and clearance mechanisms and sensitivity of the target tissue to the pollutant. These results indicate the importance of exercising caution when using data from one route of exposure to predict the toxicity resulting from another route.

When evaluating the toxicity of these metals in man, possible toxicity due to exposure routes that deliver proportionately smaller doses should not be dismissed. The relatively small proportion of the pollutant reaching the host via inhalation as compared to ingestion (Table 5) may manifest greater toxicity than its absolute concentration would suggest. While the aerosol metal concentrations

TABLE 5
COMPARISONS OF METAL CONCENTRATIONS USED IN THESE EXPERIMENTS WITH THOSE FOUND IN THE ENVIRONMENT

Metal	Concentration used[a] (μg/m^3)	Threshold limit values[b] (μg/m^3)	Human intake[c] (μg/day)	
			Air	Water
Cd	200	200	7.4	160
Ni	250	1000	2.36	600

[a] Lowest concentration tested which caused an effect.
[b] American Conference of Industrial Hygenists. 1971.
[c] Schroeder *et al.*, 1970.

used in these experiments exceed those found in ambient air, they are still at or below the threshold limit value (TLV) of these metals. Considering the importance of the immune system and the ubiquitous nature of pollutants, it is important to continue investigations with Cd, Ni, and other metals.

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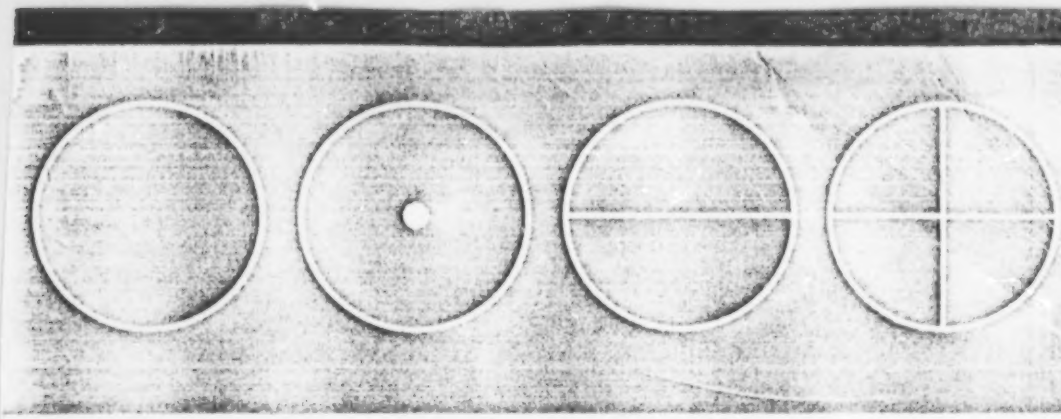

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